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Association of *eNOS* and *MCP-1* Genetic Variants with Type 2 Diabetes and Diabetic Nephropathy Susceptibility: A Case–Control and Meta-Analysis Study

Priyanka Raina, et al. *[full author details at the end of the article]*

Abstract

Type 2 diabetes (T2D) and its secondary complications result from the complex interplay of genetic and environmental factors. To understand the role of these factors on disease susceptibility, the present study was conducted to assess the association of *eNOS* and *MCP-1* variants with T2D and diabetic nephropathy (DN) in two ethnically and geographically different cohorts from North India. A total of 1313 subjects from two cohorts were genotyped for *eNOS* (rs2070744, rs869109213 and rs1799983) and *MCP-1* (rs1024611 and rs3917887) variants. Cohort-I (Punjab) comprised 461 T2D cases (204 T2D with DN and 257 T2D without DN) and 315 healthy controls. Cohort-II (Jammu and Kashmir) included 337 T2D (150 T2D with DN and 187 T2D without DN) and 200 controls. Allele, genotype and haplotype frequencies were compared among the studied participants, and phenotype–genotype interactions were determined. Meta-analysis was performed to investigate the association between the selected variants and disease susceptibility. All three *eNOS* variants were associated with 1.5–4.0-fold risk of DN in both cohorts. *MCP-1* rs1024611 conferred twofold risk towards DN progression in cohort-II, while rs3917887 provided twofold risk for both T2D and DN in both cohorts. *eNOS* and *MCP-1* haplotypes conferred risk for T2D and DN susceptibility. Phenotype–genotype interactions showed significant associations between the studied variants and anthropometric and biochemical parameters. In meta-analysis, all *eNOS* variants conferred risk towards DN progression, whereas no significant association was observed for *MCP-1* rs1024611. We show evidences for an association of *eNOS* and *MCP-1* variants with T2D and DN susceptibility.

Keywords Genetic variant · Type 2 diabetes · Diabetic nephropathy · eNOS · MCP-1

Introduction

Endothelial dysfunction and chemotaxis have been involved in the pathogenesis of renal microvascular complications in patients with and without diabetes (Karalliedde and Gnudi 2011; Awad et al. 2015; Murkamilov et al. 2017). Under normal physiological conditions, there is a balanced release of endothelium-derived vasodilator factors, but this balance is altered in diabetes mellitus which contributes to the progression of vascular and organ damage (Dhananjayan et al. 2016). Chemotaxis plays a pivotal role in the enrolment of leucocytes to inflammation and infection sites (Jin et al. 2008). This accumulation of leucocytes in various tissues is a pathologic hallmark of both type 2 diabetes (T2D) and its related complication such as diabetic nephropathy (DN), which results in altered production of cytokines, reactive oxygen species and various proteases (Galkina and Ley 2006; Tesch 2010).

Nitric oxide (NO) is produced mainly by endothelial nitric oxide synthase (eNOS) enzyme by converting L-arginine to L-citrulline (Komers and Anderson 2003; Förstermann and Münzel 2006). Any impairment in the activity of eNOS enzyme contributes to insulin resistance, diabetes and chronic renal failure (Komers and Anderson 2003; Rask-Madsen and King 2007; Dhananjayan et al. 2016). The *eNOS* gene variants may influence eNOS expression, which may lead to NO abnormalities (Ahluwalia et al. 2008; Li and Takahashi 2012; da Silva et al. 2018). Many studies have observed that the *eNOS* gene variants rs2070744 (-786 T>C, promoter polymorphism), rs869109213 (4a/b, 27 bp repeat in intron 4) and rs1799983 (894 G>T, exon 7: 298Asp to Glu substitution) were associated with different stages of DN (Noiri et al. 2002; Mehrab-Mohseni et al. 2011; Dellamea et al. 2014; Huo et al. 2015). The functional effect of these variants is to reduce mRNA expression or alter eNOS functions, which leads to reduction in NO production (Tsukada et al. 1998; Nakayama et al. 1999; Tesauro et al. 2000).

Monocyte chemoattractant protein-1 (MCP-1) is one of the first discovered and most widely studied chemokine (Van Coillie et al. 1999; Panee 2012). MCP-1 is secreted by endothelial cells, monocytes, fibroblasts, vascular smooth muscle cells and T cells (Conti and DiGioacchino 2001; Deshmane et al. 2009). MCP-1 activates monocytes chemotaxis and transendothelial migration to inflammation sites by interacting with the C–C chemokine receptor 2 (CCR2) in monocytes (Mackay 1996; O'Hayre et al. 2008). Hyperglycemia stimulates MCP-1 secretion from kidney cells, which results in tubular macrophage and myofibroblast accumulation, and finally causes tubular injury and renal fibrosis (Morii et al. 2003; Tesch 2008; Jing et al. 2011). In diabetic and nephropathy patients, the MCP-1 serum levels have been shown to be influenced by genetic variants of *MCP-1* gene. Specifically, rs1024611 (Promoter, −2518 A>G) and rs3917887 (Intron 1, 14 bp insertion/deletion, int1del554-567) polymorphisms have been shown to affect MCP-1 expression (Rovin and Saxena 1999; Fenoglio et al. 2004; Del Guerra et al. 2010). Several previous studies have reported a positive association of rs1024611 with T2D and DN susceptibility (Simeoni et al. 2004; Ahluwalia et al. 2009; Jing et al. 2011; Raina et al. 2015a). However, in the case of *MCP-1* rs3917887, only one study (Ahluwalia et al. 2009) has reported significant association with T2D and DN susceptibility.

The genetic architecture and environment factors can significantly influence the resulting disease phenotype. Due to ethnic and genetic differences, the same variant can have a heterogeneous effect in two groups (Lin et al. 2007). Individual genetic variant may not directly show association with disease manifestation but under the influence of other disease associated variants found within a same gene or in related genes can demonstrate disease association, emphasizing the combined effect of genetic variants on the disease manifestation (Liu et al. 2008). Genetic variations in *eNOS* and *MCP-1* genes play a pivotal role in endothelial dysfunction, chemotaxis and inflammation (Ahluwalia et al. 2009). Previous studies from India have investigated the role of these variants among T2D and DN (Ahluwalia et al. 2008, 2009; Tiwari et al. 2009; Cheema et al. 2013) but so far, no study has been conducted in India to elucidate the role of these variants among the end-stage renal disease (ESRD) evolved from T2D. In addition, most of these previous studies have only compared T2D cases with DN rather than healthy controls, which can provide inconclusive associations. Furthermore, DN patients enrolled were from different stages of nephropathy (Joo et al. 2007; Ahluwalia et al. 2008, 2009; Jafari et al. 2011; Santos et al. 2011; Shoukry et al. 2012; Narne et al. 2014; Huo et al. 2015). Therefore, the present study recruited only last-stage nephropathy cases with ESRD and compared them with T2D cases (without any other microvascular and macrovascular complications) as well as with healthy controls to obtain reliable results. Our study also aimed to fill the gap in the number of genetic association studies of *MCP-1* and *eNOS* genes among the selected two North Indian populations, which have different dietary habits, climatic conditions and ethnical origins.

Material and Methods

Study Population

This case–control study enrolled 1313 subjects from two ethnically and geographically different cohorts from North India. From the population of Punjab (Cohort-I), 776 samples were collected comprising 204 T2D with DN cases, 257 T2D without DN cases and 315 healthy controls. A total of 537 samples were collected from the population of Jammu and Kashmir (Cohort-II) involving 150 T2D with DN, 187 T2D without DN and 200 healthy controls. A prior written informed consent was obtained as per the Indian Council of Medical Research guidelines from each participant recruited in the study. The study was approved by the Ethics Committee of Guru Nanak Dev University, Punjab, India.

Subject Inclusion and Exclusion Criteria

T2D Without DN Group

T2D cases were recruited according to criterion given by American Diabetic Association (2011). This group included T2D cases without any microvascular or

macrovascular complications and this inclusion criterion was achieved by performing the biochemical tests, obtaining the clinical information of the enrolled cases from the clinicians and collaborated hospitals, and by extracting the information from the proforma filled by the patients. Cases with Type 1 diabetes, gestational diabetes mellitus, and any other type of diabetes were excluded from the study. Enrolled T2D cases had median age of 56 years [Interquartile range (IQR), 48–63 years] in cohort-I, and 52 years (IQR, 47–60 years) in cohort-II. A total of 115 (45%) males and 142 (55%) females were recruited in cohort-I, while cohort-II comprised 110 males (59%) and 77 (41%) females.

T2D with DN Group

All T2D with DN patients were with ESRD and were undergoing haemodialysis. These ESRD cases had T2D as a primary cause of kidney damage, and patients with hypertension, acute kidney injury, nephrotic syndrome, glomerulonephritis and systemic lupus erythematosus were excluded from the study. The minimum duration of T2D in these patients at the time of sample collection was 5 years. ESRD cases enrolled in this study were referred by the nephrologists of the collaborating hospitals according to the criterion given by Levey et al. (2011). These patients had less than 10% kidney function, > 300 mg/g albumin-to-creatinine ratio, < 15 ml/min/1.73m2 glomerular filtration rate and uremia. Cases with other microvascular and macrovascular complications such as diabetic retinopathy, neuropathy and cardiovascular diseases were excluded from the study. The median age of DN cases enrolled in cohort-I was 60 years (IQR, 55–65 years), and 61 years (56–65 years) in cohort-II. In addition, cohort-I comprised 136 (67%) males and 68 (33%) females, while cohort-II comprised 106 (71%) males and 44 (29%) females.

Control group

The controls enrolled were healthy individuals with no medical history of T2D. Controls were gender and ethnicity matched, and above 40 years of age. Subjects with family history of T2D among first-degree relatives were excluded. The controls recruited had a median age of 44 years (IQR, 45–62 years) in cohort-I, and 52 years (IQR, 46–60 years) in cohort-II. In addition, cohort-I comprised 124 (39%) males and 191 (61%) females, while cohort-II comprised 113 (56%) males and 87 (44%) females.

Clinical Data Analysis

The protocols used for anthropometric measurements and biochemical analyses have been explained previously (Raina et al. 2015b).

Genetic Analysis

The salting out method was used for genomic DNA extraction from blood lymphocytes (Miller et al. 1988). Five genetic variants of *MCP-1* (rs1024611 and

rs3917887) and *eNOS* genes (rs2070744, rs1799983 and rs869109213) were selected based on comprehensive literature review and information available in the dbSNP database (Joo et al. 2007; Ahluwalia et al. 2009; Tiwari et al. 2009; Shoukry et al. 2012; Narne et al. 2014; Huo et al. 2015). Moreover, these five polymorphisms were observed to influence the pathogenesis of T2D and its secondary complications by a range of mechanisms (Rovin and Saxena 1999; Rask-Madsen and King 2007; Ahluwalia et al. 2009; da Silva et al. 2018).

The primer sequences and PCR conditions used for *MCP-1* (rs1024611) and *eNOS* (rs1799983 and rs869109213) were based on the published literatures (Little 2001; Colombo et al. 2003; Zhang et al. 2005; Bucova el al. 2009; Kincl et al. 2009). The primers for *eNOS* rs2070744 and *MCP-1* rs3917887 were designed using Primer3. Restriction fragment length polymorphism (RFLP) and amplification-refractory mutation system (ARMS) PCR assays were used for genotyping. Details of the PCR and restriction enzyme assays are given in Supplementary Table 1.

Statistical Analysis

SPSS version 20.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Genetic Data Analysis

The minor allele frequency and Hardy–Weinberg Equilibrium (HWE) of the studied variants were determined using the Haploview software (Version 4.2). Four genetic models (the allelic, the recessive, the dominant and the codominant) were used to evaluate the risk of the selected genetic variants on disease susceptibility. These genetic models were assessed using the chi-squared test and Odds ratios (OR) along with a 95% confidence interval (CI). The p values were corrected for the effect of confounding factors such as age, gender, body mass index (BMI) and waist-to-hip ratio (WHR) by applying logistic regression models. The p value < 0.05 was considered significant. CaTS power calculator was used to calculate the post hoc power of study (Skol et al. 2006).

Haplotype Analysis

HaploView software (Version 4.2) was used to determine pair-wise linkage disequilibrium (LD), and haplotype frequencies with the lowest frequency threshold of 0.05 (Barrett et al. 2005). The LD-plot function of the software was used to measure LD based on the D′ (Lewontin's coefficient) value.

Genotype–Phenotype Interactions

The Kruskal–Wallis test was used to assess the association of BMI, triglycerides, cholesterol, low-density lipoprotein, high-density lipoprotein, very low-density lipoprotein, random blood sugar, urea, creatinine, systolic blood pressure and diastolic

blood pressure levels with the studied genetic variants in all samples (cohort-I plus cohort-II). The graphs were generated using GraphPad Prism (Version 8.0.1).

Meta-Analysis

The meta-analysis was performed using the web tool MetaGenyo (Martorell-Marugan et al. 2017). Forest plots were prepared for the allele contrast model to identify the individual and pooled effect of the studies included in the meta-analysis**.** Publication bias was assessed by Egger's test (significance level at $p < 0.05$). The I^2 (inconsistency) value was used to assess the heterogeneity in different studies. The pooled OR was combined using the fixed effect and random effect models.

Results

Distribution of demographic, anthropometric, clinical and biochemical parameters of cohort-I and cohort-II for the majority of parameters have been reported earlier (Raina et al. 2015b) and remaining variables for T2D with and without DN cases are given in Table 1. In both cohorts, comparison between T2D patients with and without DN revealed a significant difference in male-to-female ratio, smoking and obesity parameters. Interestingly, when patients from cohort-I were compared to patients from cohort-II, a male-to-female ratio, religion status, diet, alcohol intake, smoking, obesity, education, occupation economic and habitat statuses were found significantly different for T2D without DN cases (Table 1). Overall, patients with Hindu and Muslim religions, non-vegetarian diet, non-obese, educated (graduate or above), salaried job and living in urban areas were significantly more in cohort-II than cohort-I (Table 1).

Genotype frequencies of all *eNOS* and *MCP-1* variants in control participants were in agreement with HWE. The power of the study was more than 80% for all the studied variants in two cohorts.

Association of *eNOS* variants in T2D with DN

The minor allele frequency of all the three *eNOS* variants was higher in T2D with DN cases compared to controls in both cohorts (Table 2 and Table 3). The rs2070744 TC + CC genotypes conferred 1.7–2.0-fold risk for DN in both the studied cohorts. The rs1799983 GT + TT and GT genotypes were significantly associated with the risk of DN (1.5–1.7-fold) in both cohorts. For rs869109213, genotypes aa and ba conferred 4.2 and 1.8-fold risk towards DN in cohort-I and cohort-II, respectively (Table 3).

Association of *eNOS* Variants in T2D Without DN

Among T2D cases except for rs869109213, minor allele frequency of rs1799983 and rs2070744 was higher in cases as compared to controls (Tables 2 and 3). In

Table 1 Demographic, socioeconomic and epidemiological characteristics of T2D with DN and T2D without DN patients at the time of admission in two populations

	Cohort-I			Cohort-II			Cohort-I vs Cohort-II (T2D with DN)	Cohort-I vs Cohort-II (T2D without DN)
	T2D with DN	T2D without DN	p value	T2D with DN	T2D without DN	p value	p value	p value
Female, no. (%)	68 (33.3)	142 (55.3)	**<0.001**	44 (29.3)	77 (41.2)	0.024*	0.424	0.003*
Religion, no. (%)								
Hindu	70 (34.3)	122 (47.5)	**0.011***	103 (68.7)	141 (75.4)	NA	NA	<0.001*
Muslim	1 (0.5)	0		33 (22.0)	46 (24.6)			
Sikh	133 (65.2)	133 (51.8)		14 (9.3)	0			
Christian	0	2 (0.8)		0	0			
Diet, no. (%)[a]								
Vegetarian	107 (52.5)	168 (65.4)	**0.003***	64 (42.7)	79 (42.2)	0.938	0.056	**<0.001***
Non-vegetarian	95 (46.6)	84 (32.7)		86 (57.3)	108 (57.8)			
Alcohol use, no. (%)[b]								
Yes	45 (22.1)	39 (15.2)	0.071	2 (1.3)	64 (34.2)	**<0.001***	**<0.001***	**<0.001***
No	158 (77.4)	212 (82.5)		147 (98.0)	123 (65.8)			
Smoking, no. (%)[a]								
Yes	23 (11.3)	10 (3.9)	**0.003***	2 (1.3)	44 (23.5)	**<0.001***	**<0.001***	**<0.001***
No	179 (87.7)	242 (94.2)		148 (98.7)	143 (76.5)			
Obesity, no. (%)[c]								
Yes	130 (63.7)	247 (96.1)	**<0.001***	67 (44.7)	64 (34.2)	0.013*	**0.004***	**<0.001***
No	74 (36.3)	4 (1.6)		73 (48.7)	123 (65.8)			
Education status, no. (%)[d]								
Graduate or above	33 (16.2)	52 (20.2)	0.95	59 (39.3)	68 (36.4)	0.171	**<0.001***	**<0.001***
Secondary school or less	83 (40.7)	131 (51.0)		77 (51.3)	110 (58.8)			
Illiterate	43 (21.1)	63 (24.5)		14 (9.3)	9 (4.8)			
Occupation, no. (%)[e]								

Table 1 (continued)

	Cohort-I			Cohort-II			Cohort-I vs Cohort-II (T2D with DN)	Cohort-I vs Cohort-II (T2D without DN)
	T2D with DN	T2D without DN	p value	T2D with DN	T2D without DN	p value	p value	p value
No salary service	95 (46.6)	172 (66.9)	0.051	44 (29.3)	72 (38.5)	0.078	**<0.001***	**<0.001***
Salary service	62 (30.4)	74 (28.8)		106 (70.7)	115 (61.5)			
Economic status, no. (%)[f]								
Higher class	59 (28.9)	27 (10.5)	**<0.001***	24 (16.0)	26 (13.9)	0.605	**<0.001***	**<0.001***
Middle class	87 (42.6)	209 (81.3)		108 (72.0)	132 (70.6)			
Lower class	14 (6.9)	10 (3.9)		18 (12.0)	29 (15.5)			
Habitat status, no. (%)[g]								
Urban	121 (59.3)	137 (53.3)	**<0.001***	97 (64.7)	129 (69.0)	0.241	**<0.001***	**0.005***
Sub-urban	4 (2.0)	41 16.0)		21 (14.0)	31 (16.6)			
Rural	78 (38.2)	66 (25.7)		32 (21.3)	27 (14.4)			

Bold indicates the statistically significant values

NA not applicable, no. number, DN diabetic nephropathy, T2D Type 2 diabetes

Cohort-I: Punjab population; Cohort-II: Jammu and Kashmir population

[a] Missing data of 7 samples in Cohort-I (T2D with DN = 2; T2D without DN = 5)

[b] Missing data of 7 samples of Cohort-I (T2D with DN = 1; T2D without DN = 6) and 1 sample of Cohort-II (T2D with DN = 1)

[c] Missing data of 6 samples in Cohort-I (T2D without DN = 6) and 10 samples of Cohort-II (T2D with DN = 10)

[d] Missing data of 56 samples in Cohort-I ion (T2D with DN = 45; T2D without DN = 11)

[e] Missing data of 58 samples in Cohort-I (T2D with DN = 47; T2D without DN = 11)

[f] Missing data of 55 samples in Cohort-I (T2D with DN = 44; T2D without DN = 11)

[g] Missing data of 14 samples in Cohort-I (T2D with DN = 1; T2D without DN = 13)

p values were obtained by chi-square or Mann–Whitney U tests

*p value < 0.05 is considered significant

Table 2 Genotype and allele frequencies of *eNOS* and *MCP-1* gene variants among the two studied cohorts

	Cohort-I			Cohort-II		
	T2D with DN (n=204)	T2D without DN (n=257)	Controls (n=315)	T2D with DN (n=150)	T2D without DN (n=187)	Controls (n=200)
eNOS rs2070744						
TT	116 (56.8)	157 (61.1)	220 (69.8)	88 (58.7)	122 (65.2)	145 (72.5)
TC	83 (40.7)	94 (36.6)	90 (28.6)	56 (37.3)	60 (32.1)	50 (25.0)
CC	5 (2.5)	6 (2.3)	5 (1.6)	6 (4.0)	5 (2.7)	5 (2.5)
T	315 (77.2)	408 (79.4)	530 (84.1)	232 (77.3)	304 (81.3)	340 (85.0)
C	93 (22.8)	106 (20.6)	100 (15.9)	68 (22.7)	70 (18.7)	60 (15.0)
eNOS rs1799983						
GG	120 (58.8)	169 (65.8)	214 (67.9)	84 (56.0)	108 (57.8)	137 (68.5)
GT	77 (37.7)	82 (31.9)	96 (30.5)	60 (40.0)	73 (39.0)	58 (29.0)
TT	7 (3.5)	6 (2.3)	5 (1.6)	6 (4.0)	6 (3.2)	5 (2.5)
G	317 (77.7)	420 (81.7)	524 (83.2)	228 (76.0)	289 (77.3)	332 (83.0)
T	91 (22.3)	94 (18.3)	106 (16.8)	72 (24.0)	85 (22.7)	68 (17.0)
eNOS rs869109213						
bb	127 (62.3)	180 (70.0)	214 (67.9)	88 (58.6)	127 (67.9)	145 (72.5)
ba	65 (31.9)	72 (28.1)	95 (30.2)	55 (36.7)	55 (29.4)	50 (25.0)
aa	12 (5.9)	5 (1.9)	6 (1.9)	7 (4.7)	5 (2.7)	5 (2.5)
b	319 (78.2)	432 (84.0)	524 (83.2)	231 (77.0)	309 (82.6)	340 (85.0)
a	89 (21.8)	82 (16.0)	106 (16.8)	69 (23.0)	65 (17.4)	60 (15.0)
MCP-1 rs1024611						
AA	83 (40.7)	137 (53.3)	143 (45.4)	55 (36.6)	73 (39.0)	95 (47.5)

Table 2 (continued)

	Cohort-I			Cohort-II		
	T2D with DN ($n=204$)	T2D without DN ($n=257$)	Controls ($n=315$)	T2D with DN ($n=150$)	T2D without DN ($n=187$)	Controls ($n=200$)
AG	101 (49.5)	109 (42.4)	149 (47.3)	70 (46.7)	95 (50.8)	87 (43.5)
GG	20 (9.8)	11 (4.3)	23 (7.3)	25 (16.7)	19 (10.2)	18 (9.0)
A	267 (65.4)	383 (74.5)	435 (69.0)	180 (60.0)	241 (64.4)	277 (69.2)
G	141 (34.6)	131 (25.5)	195 (31.0)	120 (40.0)	133 (35.6)	123 (30.8)
MCP-1 rs3917887						
II	107 (52.5)	143 (55.6)	204 (64.8)	73 (48.7)	104 (55.6)	136 (68.0)
ID	91 (44.6)	97 (37.8)	93 (29.5)	64 (42.6)	71 (38.0)	56 (28.0)
DD	6 (2.9)	17 (6.6)	18 (5.7)	13 (8.7)	12 (6.4)	8 (4.0)
I	305 (74.8)	383 (74.5)	501 (79.5)	210 (70.0)	279 (74.6)	328 (82.0)
D	103 (25.2)	131 (25.5)	129 (20.5)	90 (30.0)	95 (25.4)	72 (18.0)

DN diabetic nephropathy, *T2D* Type 2 diabetes, *Cohort-I* Punjab population, *Cohort-II* Jammu and Kashmir population

Table 3 Comparison of *eNOS* gene variants among the two studied cohorts

Cohort-I

	T2D with DN vs controls	T2D without DN vs controls	T2D with DN vs T2D without DN
eNOS rs2070744			
Genotypes			
p	**0.010***	0.087	0.654
Alleles			
p	**0.005***	**0.038***	0.426
OR (95% CI)	1.56 (1.14–2.13)	1.37 (1.02–1.85)	1.14 (0.83–1.56)
Dominant model (TC+CC vs TT)			
p	**0.012****	**0.027****	0.359
OR (95% CI)	1.75 (1.12–2.57)	1.50 (1.04–2.16)	1.19 (0.82–1.72)
Recessive model (CC vs TT+TC)			
p	0.484	0.517	0.935
OR (95% CI)	1.56 (0.44–5.56)	1.49 (0.45–5.0)	1.05 (0.32–3.45)
Codominant model (TC vs TT+CC)			
p	**0.015****	**0.028****	0.367
OR (95% CI)	1.70 (1.11–2.56)	1.5 (1.05–2.17)	1.19 (0.82–1.73)
eNOS rs1799983			
Genotypes			
p	0.068	0.739	0.289
Alleles			
p	**0.027***	0.517	0.130
OR (95% CI)	1.43 (1.04–1.92)	1.11 (0.81–1.49)	1.28 (0.93–1.79)
Dominant model (GT+TT vs GG)			
p	**0.042****	0.582	0.126
OR (95% CI)	1.53 (1.02–2.31)	1.1 (0.78–1.56)	1.35 (0.92–1.96)
Recessive model (TT vs GG+GT)			
p	0.172	0.517	0.480
OR (95% CI)	2.22 (0.69–7.14)	1.49 (0.45–5.0)	1.49 (0.49–4.55)
Codominant model (GT vs GG+TT)			
p	0.086	0.713	0.190
OR (95% CI)	1.38 (0.95–2.0)	1.07 (0.75–1.53)	1.29 (0.88–1.90)
eNOS rs869109213			
Genotypes			
p	**0.022***	0.784	**0.041***
Alleles			
p	**0.044***	0.692	**0.022***
OR (95% CI)	1.37 (1.0–1.89)	0.93 (0.68–1.28)	1.47 (1.05–2.04)

Table 3 (continued)

Cohort-I

	T2D with DN vs controls	T2D without DN vs controls	T2D with DN vs T2D without DN
Dominant model (ba + aa vs bb)			
p	0.183	0.589	0.079
OR (95% CI)	1.28 (0.88–1.85)	0.91 (0.63–1.30)	1.41 (0.96–2.08)
Recessive model (aa vs bb + ba)			
p	**0.011****	0.745	0.071
OR (95% CI)	4.20 (1.38–12.7)	1.23 (0.35–4.35)	2.90 (0.9–8.9)
Codominant model (ba vs bb + aa)			
p	0.739	0.520	0.369
OR (95% CI)	1.07 (0.73–1.56)	0.89 (0.62–1.28)	1.20 (0.80–1.79)

Cohort-II

	T2D with DN vs controls	T2D without DN vs controls	T2D with DN vs T2D without DN
eNOS rs2070744			
Genotypes			
p	**0.025***	0.293	0.429
Alleles			
p	**0.009***	0.167	0.207
OR (95% CI)	1.67 (1.14–2.44)	1.30 (0.89–1.89)	1.27 (0.88–1.85)
Dominant model (TC + CC vs TT)			
p	**0.012****	0.123	0.216
OR (95% CI)	2.0 (1.2–3.3)	1.41 (0.91–2.13)	1.32 (0.85–2.04)
Recessive model (CC vs TT + TC)			
p	0.426	0.914	0.496
OR (95% CI)	1.85 (1.19–2.94)	1.08 (0.30–3.70)	1.52 (0.45–5.0)
Codominant model (TC vs TT + CC)			
p	**0.014****	0.123	0.314
OR (95% CI)	2.0 (1.15–3.4)	1.42 (0.91–2.21)	1.26 (0.80–1.98)
eNOS rs1799983			
Genotypes			
p	0.055	0.090	0.900
Alleles			
p	**0.022***	**0.045***	0.698
OR (95% CI)	1.54 (1.06–2.22)	1.43 (1.01–2.04)	0.93 (0.65–1.33)

Table 3 (continued)

Cohort-II

	T2D with DN vs controls	T2D without DN vs controls	T2D with DN vs T2D without DN
Dominant model (GT+TT vs GG)			
p	**0.016****	**0.028****	0.747
OR (95% CI)	1.71 (1.0–2.65)	1.60 (1.05–2.43)	0.93 (0.60–1.44)
Recessive model (TT vs GG+GT)			
p	0.426	0.675	0.697
OR (95% CI)	1.61 (0.49–5.43)	1.30 (0.39–4.35)	0.80 (0.25–2.52)
Codominant model (GT vs GG+TT)			
p	**0.031****	**0.036****	0.857
OR (95% CI)	1.64 (1.04–2.56)	1.58 (1.03–2.42)	1.04 (0.67–1.62)
eNOS rs869109213			
Genotypes			
p	**0.023***	0.608	0.184
Alleles			
p	**0.007***	0.368	0.069
OR (95% CI)	1.69 (1.15–2.5)	1.19 (0.81–1.75)	1.43 (0.97–2.08)
Dominant model (ba+aa vs bb)			
p	**0.035****	0.325	1.49 (0.95–2.33)
OR (95% CI)	1.73 (1.04–2.9)	1.25 (0.81–1.92)	
Recessive model (aa vs bb+ba)			
p	0.270	0.914	0.327
OR (95% CI)	1.92 (0.60–6.25)	1.08 (0.30–3.70)	1.79 (0.55–5.88)
Codominant model (ba vs bb+aa)			
p	**0.030****	0.324	1.39 (0.88–2.20)
OR (95% CI)	1.79 (1.06–3.0)	1.25 (0.81–1.92)	

Bold indicates the statistically significant values

DN diabetic nephropathy, *T2D* Type 2 diabetes, *Cohort-I* Punjab population, *Cohort-II* Jammu and Kashmir population

*p value < 0.05 is considered significant; *OR* odds ratio, *CI* confidence interval

**p value corrected for age, gender, BMI and WHR

cohort-I, rs2070744 TC+CC and TC genotypes were significantly associated with 1.5-fold T2D risk. No significant association of rs2070744 genotypes was observed in cohort-II. The rs1799983 GT+TT and GT genotypes were significantly associated with T2D (1.6-fold) risk in cohort-II, while in cohort-I no association was

observed. For rs869109213, no significant association was observed with T2D in both cohorts (Table 3).

Association of *MCP-1* Variants in T2D with DN

Frequency of rs1024611 G allele was higher in T2D with DN cases from both cohorts compared to T2D without DN and controls (Tables 2 and 4). *MCP-1* rs1024611 G allele provided a risk towards DN in both cohorts. In cohort-I, rs1024611 genotypes AG + GG and GG conferred approximately 1.5–2.6-fold risk for DN. In cohort-II, rs1024611 AG + GG genotypes provided 1.7-fold risk towards DN. For rs3917887, ID + DD genotypes conferred 1.8–2.0-fold risk for DN in both cohorts (Table 4).

Association of *MCP-1* Variants in T2D Without DN

MCP-1 rs1024611 G allele provided a protection for T2D development in cohort-I. However, no significant association was observed in cohort-II. In the case of *MCP-1* rs3917887, ID + DD and ID genotypes provided 1.5–1.7-fold risk towards T2D development in both cohorts (Table 4).

Association of *eNOS* Haplotypes

The frequency of haplotype T-b-G was significantly higher in controls in comparison to DN cases and provided 1.6-fold protection towards DN in both cohorts. In cohort-I, C-b-T haplotype gave 2.9-fold risk for DN progression. In cohort-II, haplotype C-a-T (with all variant alleles) conferred 3.3–3.9-fold risk towards T2D and DN (Table 5). Based on the measure of LD, no significant results were observed between the *eNOS* variants in both cohorts ($D' < 0.5$).

Association of *MCP-1* Haplotypes

In *MCP-1* haplotype distribution, the frequency of haplotype G-D (containing variant alleles) was significantly higher in DN group compared to other groups. G-D haplotype provided 2.0–3.8-fold risk for DN and 1.6–2.7-fold risk for T2D progression in both cohorts. Haplotype A-I conferred a protection against DN in cohort-II, while haplotype G-I provided a protection towards T2D development in cohort-I (Table 5). The selected variants of *MCP-1* were not in LD in both cohorts ($D' < 0.5$).

Genotype–Phenotype Interactions

The phenotype–genotype interaction was carried out to understand the effect of observed genotypes of *eNOS* and *MCP-1* genetic variants on the different covariates presented previously (Raina et al. 2015b) and in this paper. Among all the variants tested in this study, the *eNOS* rs1799983 (TT) genotype was associated with lower

Table 4 Comparison of *MCP-1* gene variants among the two studied cohorts

Cohort-I

	T2D with DN vs controls	T2D without DN vs controls	T2D with DN vs T2D without DN
MCP-1 rs1024611			
Genotypes	0.429	0.094	**0.006***
p			
Alleles			
p	0.225	**0.042***	**0.003***
OR (95% CI)	1.18 (0.90–1.54)	0.76 (0.59–0.99)	1.54 (1.16–2.04)
Dominant model (AG+GG *vs* AA)			
p	0.290	0.059	**0.041****
OR (95% CI)	1.20 (0.85–1.72)	0.73 (0.52–1.01)	1.54 (1.02–2.32)
Recessive model (GG *vs* AA+AG)			
p	0.312	0.128	**0.026****
OR (95% CI)	1.39 (0.74–2.56)	0.57 (0.27–1.19)	2.55 (1.12–5.82)
Codominant model (AG *vs* AA+GG)			
p	0.623	0.242	0.129
OR (95% CI)	1.09 (0.77–1.55)	0.82 (0.59–1.14)	1.33 (0.92–1.93)
MCP-1 rs3917887			
Genotypes			
p	**0.001***	0.08	0.100
Alleles			
p	0.072	**0.044***	0.933
OR (95% CI)	1.32 (0.98–1.75)	1.33 (1.01–1.75)	0.99 (0.73–1.33)
Dominant model (ID+DD *vs* II)			
p	**0.004****	**0.028****	0.495
OR (95% CI)	1.80 (1.20–2.70)	1.48 (1.04–2.09)	1.14 (0.79–1.64)
Recessive model (DD *vs* II+ID)			
p	0.142	0.655	0.072
OR (95% CI)	0.50 (0.19–1.28)	1.16 (0.59–2.33)	0.43 (0.17–1.11)
Codominant model (ID *vs* II+DD)			
p	**0.001****	0.055	0.136
OR (95% CI)	1.97 (1.30–2.98)	1.42 (0.99–2.04)	1.33 (0.91–1.93)

Table 4 (continued)

Cohort-II			
	T2D with DN vs controls	T2D without DN vs controls	T2D with DN vs T2D without DN
MCP-1 rs1024611			
Genotypes			
p	**0.036***	0.243	0.211
Alleles			
p	**0.011***	0.155	0.237
OR (95% CI)	1.49 (1.10–2.04)	1.25 (0.92–1.67)	1.20 (0.88–1.64)
Dominant model (AG+GG *vs* AA)			
p	**0.029****	0.093	0.656
OR (95% CI)	1.75 (1.06–2.87)	1.41 (0.94–2.13)	1.11 (0.71–1.72)
Recessive model (GG *vs* AA+AG)			
p	0.089	0.698	0.078
OR (95% CI)	1.93 (0.91–4.09)	1.15 (0.58–2.27)	1.75 (0.93–3.33)
Codominant model (AG *vs* AA+GG)			
p	0.556	0.150	0.450
OR (95% CI)	1.14 (0.74–1.74)	1.34 (0.90–2.0)	0.85 (0.55–1.30)
MCP-1 rs3917887			
Genotypes			
p	**0.001***	**0.041***	0.408
Alleles			
p	**0.0002***	**0.012***	0.184
OR (95% CI)	1.96 (1.37–2.78)	1.56 (1.10–2.17)	1.27 (0.89–1.75)
Dominant model (ID+DD *vs* II)			
p	**0.006****	**0.013****	0.204
OR (95% CI)	2.03 (1.23–3.33)	1.69 (1.12–2.56)	1.32 (0.86–2.04)
Recessive model (DD *vs* II+ID)			
p	0.069	0.283	0.434
OR (95% CI)	2.27 (0.92–5.56)	1.64 (0.66–4.17)	1.39 (0.61–3.13)
Codominant model (ID *vs* II+DD)			
p	**0.032****	**0.038****	0.382
OR (95% CI)	1.75 (1.05–2.92)	1.57 (1.03–2.41)	1.22 (0.78–1.88)

Bold indicates the statistically significant values

DN diabetic nephropathy, *T2D* Type 2 diabetes, *Cohort-I* Punjab population, *Cohort-II* Jammu and Kashmir population

**p* value < 0.05 is considered significant; *OR* odds ratio, *CI* confidence interval

***p* value corrected for age, gender, BMI and WHR

Table 5 Haplotype frequency distribution of *eNOS* and *MCP-1* gene variants

Cohort-I

Haplotype[a]	T2D with DN (*n*=204)	T2D without DN (*n*=257)	Controls (*n*=315)	T2D with DN vs Controls		T2D without DN vs Controls	
				p	OR (95% CI)	*p*	OR (95% CI)
eNOS							
T-b-G	0.50	0.59	0.61	**0.0004***	0.63 (0.49–0.81)	0.340	0.89 (0.70–1.13)
T-b-T	0.11	0.10	0.10	0.639	1.10 (0.74–1.65)	0.920	0.98 (0.67–1.44)
T-a-G	0.12	0.08	0.10	0.170	1.3 (0.88–1.94)	0.532	0.88 (0.58–1.32)
C-b-G	0.11	0.13	0.10	0.386	1.19 (0.79–1.78)	0.156	1.31 (0.90–1.89)
C-b-T	0.06	0.03	0.02	**0.002***	2.87 (1.44–5.73)	0.141	1.88 (0.80–4.42)
MCP-1							
A-I	0.52	0.58	0.55	0.460	0.91 (0.71–1.17)	0.231	0.86 (0.68–1.10)
G-I	0.23	0.16	0.25	0.376	0.88 (0.65–1.17)	**0.0004***	0.59 (0.44–0.79)
A-D	0.13	0.16	0.14	0.649	0.92 (0.64–1.32)	0.366	0.82 (0.55–1.17)
G-D	0.12	0.10	0.06	**0.001***	2.05 (1.32–3.18)	**0.044***	1.64 (1.08–2.49)

Cohort-II

Haplotype[a]	T2D with DN (*n*=150)	T2D without DN (*n*=187)	Controls (*n*=200)	T2D with DN vs Controls		T2D without DN vs Controls	
				p	OR (95% CI)	*p*	OR (95% CI)
eNOS							
T-b-G	0.51	0.57	0.62	**0.002***	0.62 (0.46–0.84)	0.170	0.82 (0.61–1.09)
T-b-T	0.13	0.12	0.11	0.620	1.12 (0.71–1.77)	0.671	1.11 (0.71–1.72)
T-a-G	0.12	0.09	0.09	0.196	1.38 (0.85–2.24)	0.794	0.95 (0.58–1.55)
C-b-G	0.11	0.11	0.10	0.924	1.01 (0.62–1.65)	0.960	1.02 (0.65–1.61)
C-b-T	0.05	0.04	0.01	**0.006***	3.87(1.38–10.86)	**0.023***	3.30 (1.19–9.17)
MCP-1							
A-I	0.48	0.51	0.57	**0.021***	0.70(0.52–0.95)	0.189	0.83 (0.62–1.10)
G-I	0.22	0.23	0.25	0.329	0.84(0.59–1.19)	0.382	0.87 (0.63–1.21)

Table 5 (continued)

Cohort-II

Haplotype[a]	T2D with DN (n=150)	T2D without DN (n=187)	Controls (n=200)	T2D with DN vs Controls		T2D without DN vs Controls	
				p	OR (95% CI)	p	OR (95% CI)
A-D	0.12	0.13	0.12	0.864	0.95(0.60–1.51)	0.964	1.00 (0.66–1.53)
G-D	0.18	0.13	0.06	**1.55×10^{-7}**	3.77(2.24–6.35)	**0.0002***	2.65 (1.55–4.52)

Bold indicates the statistically significant values

Order of SNPs-*eNOS* haplotype: rs2070744, rs869109213, rs1799983 and *MCP-1* haplotype: rs1024611 and rs3917887

DN diabetic nephropathy, *T2D* Type 2 diabetes, *Cohort-I* Punjab population, *Cohort-II* Jammu and Kashmir population

Haplotypes with ≥ 5% frequency in at least one of the three groups are presented

*$p < 0.05$ is considered significant; *OR* odds ratio, *CI* confidence interval

and higher median of BMI and creatinine levels, respectively, compared to GG and GT genotypes of rs1799983 variant (Fig. 1). *eNOS* rs2070744 (CC) genotype was associated with higher median of urea levels than TT and TC genotypes (Fig. 1). However, in the case of *MCP-1* rs3917887, II genotype was significantly associated with lower median of random sugar levels compared to DD and ID genotypes of the rs3917887 variant (Fig. 1).

Meta-Analysis

The results of the meta-analysis for the associations of *eNOS* and *MCP-1* variants with T2D and DN susceptibility are depicted in Fig. 2. Due to the fact that *MCP-1* rs3917887 had a limited number of studies, it was excluded from the meta-analysis. In T2D with DN cases, all *eNOS* variants conferred risk towards DN progression under the allele contrast model (Fig. 2, Table 6). In T2D without DN cases, *eNOS* rs2070744 and rs869109213 increased risk for T2D (Fig. 3, Table 6). For *MCP-1* rs1024611 variant, no significant association was observed for both T2D and DN. The significant heterogeneity was observed only in the case of *MCP-1* rs1024611, which could be due to less number of studies included in the meta-analysis. No publication bias was observed for any variant in the allele contrast model. The studies included in the meta-analysis are given in Supplementary Table 2.

Discussion

To our knowledge, this is the first study to decipher the association of *eNOS* (rs2070744, rs1799983 and rs869109213) and *MCP-1* (rs3917887 and rs1024611) variants with DN (ESRD) in T2D patients from both the studied cohorts.
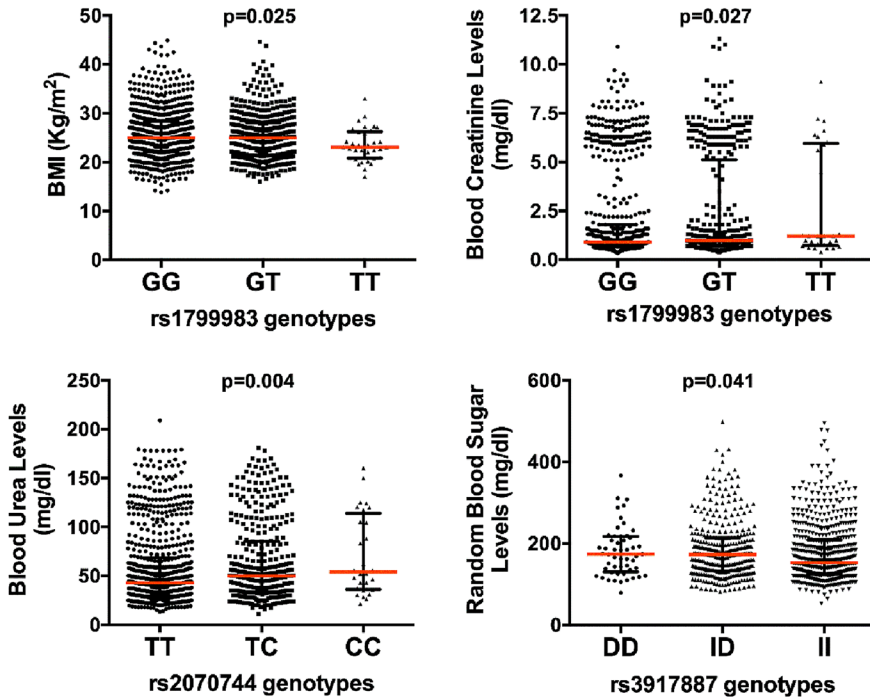
Fig. 1 Genotype–phenotype interaction analysis. Body mass index (BMI), urea, creatinine and random sugar levels were compared between different genotypes of *eNOS* and *MCP-1* gene variants. T bars represent median (in red) and Interquartile Ranges [Data not available for samples: BMI (cohort-I = 15), urea (cohort-I = 181; cohort-II = 1), creatinine (cohort-I = 202; cohort-II = 2) and random sugar (cohort-I = 191; cohort-II = 150)]

Dysfunctional eNOS and MCP-1 plays an essential role in the pathways involved in the pathogenesis of diabetes and its vascular complications (Noiri et al. 2002; Tesch 2008; da Silva et al. 2018). Genetic variants in *eNOS* and *MCP-1* genes are shown to affect their activity and may also promote DN progression in diabetic patients (Ahluwalia et al. 2009; Bagci et al. 2015; Elsisy et al. 2016). Here, we report the association of *eNOS* and *MCP-1* genetic variants with T2D and DN susceptibility in two geographically and ethnically diverse cohorts. Some genetic variants were associated with T2D susceptibility but not with the risk of DN, while some variants were not associated with T2D but in diabetic milieu increased the risk of having nephropathy. The two selected cohorts showed a significant difference in the distribution of their demographic, socioeconomic and epidemiological parameters among cases, which confirmed the fact that the two selected cohorts were different in terms of participant's characteristics. This observation was further supported by the phenotype–genotype interactions where a significant difference in the effect of the studied variants on anthropometric and biochemical parameters-related phenotype was observed. The differences observed in terms of association of these genetic variations among the two cohorts lay emphasis on the effect of geographical variation, environmental factors and ethnicity on these
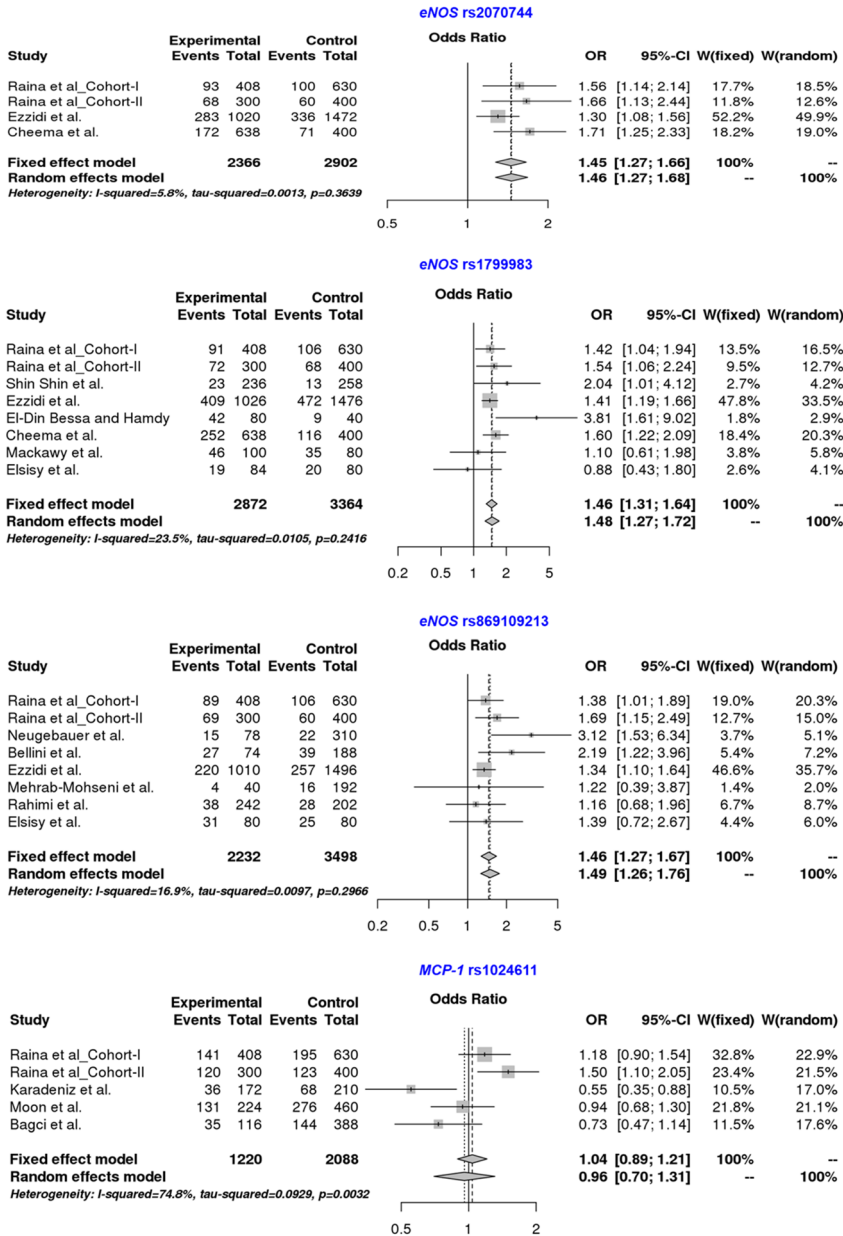
Fig. 2 Forest plot depicting association of *eNOS* (rs2070744, rs1799983, rs8691092123) and *MCP-1* (rs1024611) polymorphisms with DN susceptibility. The area of the square is proportional to the study's weight. The horizontal line represents a 95% CI. The overall effect is illustrated as diamonds with the lateral points showing CI. Experimental: DN cases; Control: Healthy controls; Events: Allele contrast model (A vs a); W: Weight; CI: Confidence interval. Raina et al_Cohort-I represents Punjab population and Raina et al_Cohort-II represents Jammu and Kashmir population

Table 6 Meta-analysis of *eNOS* and *MCP-1* variants based on subgroup analysis

	No. of studies	Test of association			Test of heterogeneity		Publication bias
		OR (95% CI)	p^a	Model	p^b	I^2	p (Egger's test)
T2D with DN							
eNOS rs2070744	4	1.45 (1.27–1.66)	**3.31E^{-08}***	Fixed	0.3639	0.0583	0.0589
eNOS rs1799983	8	1.46 (1.31–1.64)	**1E^{-10}***	Fixed	0.2416	0.2355	0.5859
eNOS rs869109213	8	1.46 (1.27–1.67)	**0.00001***	Fixed	0.2966[†]	0.1691	0.2621
MCP-1 rs1024611	5	0.96 (0.70–1.31)	0.7826	Random	**0.0032*[†]**	0.7482	0.0929
T2D without DN							
eNOS rs2070744	4	1.19 (1.04–1.37)	**0.011***	Fixed	0.5293	0	0.7408
eNOS rs1799983	8	1.13 (1.0–1.27)	0.0498	Fixed	0.6479	0	0.9148
eNOS rs869109213	7	1.29 (1.11–1.48)	**0.0006***	Fixed	0.1404	0.378	0.8045
MCP-1 rs1024611	4	0.95 (0.65–1.40)	0.804	Random	**0.002*[†]**	0.7966	0.7294

DN diabetic nephropathy, *T2D* Type 2 diabetes

[†]In the case of heterogeneity p value (p^b) < 0.1, Random effect model is used, otherwise, fixed effect model will be used instead

*p value < 0.05 is considered significant; *OR* odds ratio, *CI* confidence interval

p^a = Test of association p value; p^b = heterogeneity p value

selected variants, and this may lead to a difference towards the disease progression in the two cohorts (Tiwari et al. 2009; Raina et al. 2015b).

The *eNOS* promoter variant rs2070744 C allele is associated with reduced eNOS activity because DNA binding protein (replication protein A1) can bind only to the *eNOS* rs2070744 C allele isoform, and this results in approximately 50% reduction in *eNOS* gene transcription, which decreases eNOS protein expression and serum NO levels (Taverna et al. 2005). In cohort-I, rs2070744 was observed to be associated with T2D and DN risk. However, in cohort-II, rs2070744 conferred a risk only towards DN and no significant differences were observed for T2D without DN cases. Despite having known evidences of endothelial dysfunction and reduced NO production among T2D and DN patients with *eNOS* gene variants, a few limited studies have examined the relationship of *eNOS* rs2070744 with DN in populations such as Egyptian (Shoukry et al. 2012), Tunisian (Ezzidi et al. 2008) and Indian (Ahluwalia et al. 2008; Cheema et al. 2013), and they also observed that rs2070744 C allele is associated with DN risk. In contrast, some studies revealed no association of rs2070744 with DN (Tiwari
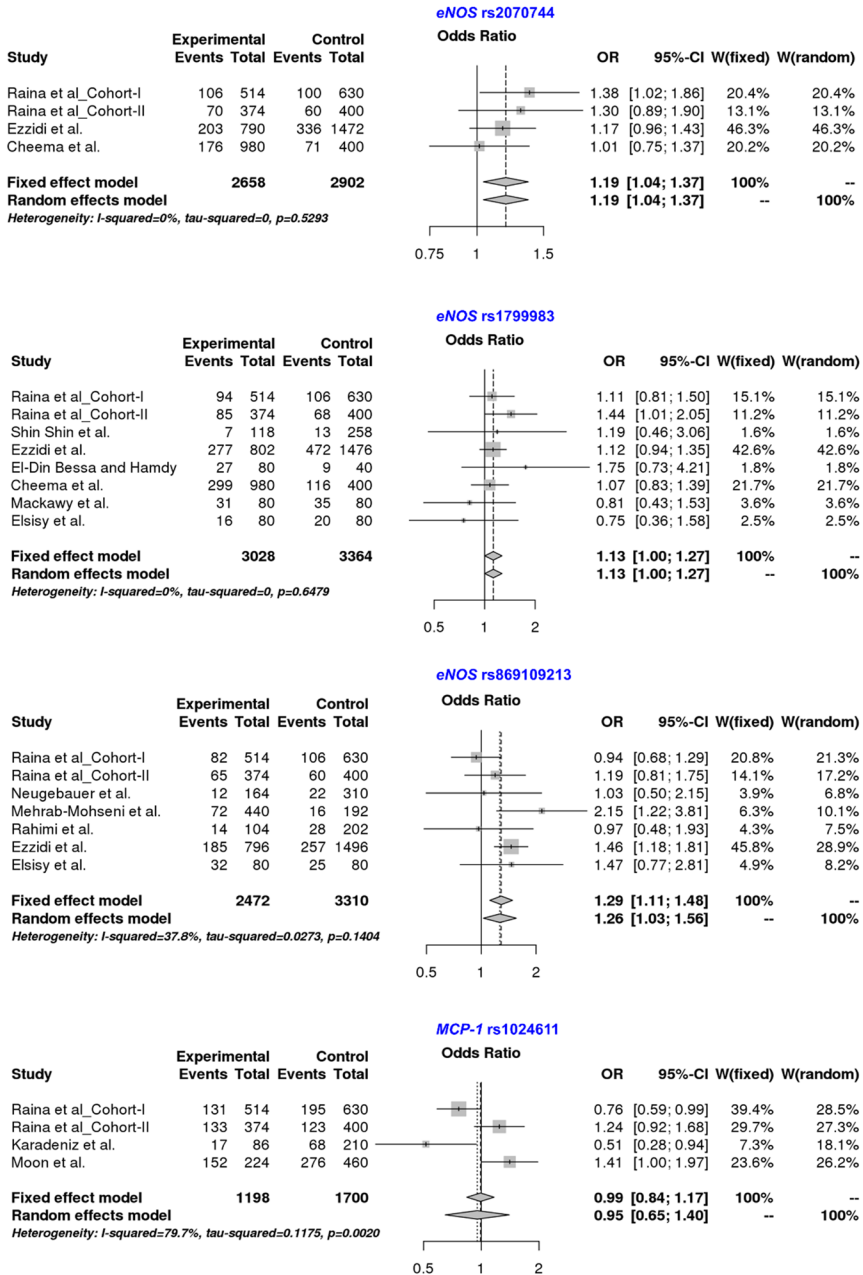
Fig. 3 Forest plot depicting association of *eNOS* (rs2070744, rs1799983, rs8691092123) and *MCP-1* (rs1024611) polymorphisms with T2D susceptibility. The area of the square is proportional to the study's weight. The horizontal line represents a 95% CI. The overall effect is illustrated as diamonds with the lateral points showing CI. Experimental: DN cases; Control: Healthy controls; Events: Allele contrast model (A vs a); W: Weight; CI: Confidence interval. Raina et al_Cohort-I represents Punjab population and Raina et al_Cohort-II represents Jammu and Kashmir population

et al. 2009; Santos et al. 2011; Narne et al. 2014; Huo et al. 2015) (Supplementary Table 3).

The rs1799983 polymorphism is believed to change the eNOS protein sequence, which leads to defective enzyme activity and degradation (Brouet et al. 2001; Costacou et al. 2006). This variant is also believed to control the intracellular distribution of eNOS and its interaction with proteins that facilitate its degradation (Brouet et al. 2001). In our study, rs1799983 T allele was significantly associated with DN in cohort-I and both T2D and DN in cohort-II. Similar results were observed in Japanese (Noiri et al. 2002), Korean (Shin Shin et al. 2004), Tunisian (Ezzidi et al 2008), Egyptian (El-Din Bessa and Hamdy 2011; Shoukry et al. 2012) and Indian populations (Ahluwalia et al. 2008; Cheema et al. 2013; Naren et al. 2014). However, in the Chinese population (Huo et al. 2015), rs1799983 G allele was conferring a risk towards DN. The Brazilian (Santos et al. 2011), Iranian (Jafari et al. 2011), Saudi Arabian (Mackawy et al. 2014) and Egyptian populations (Elsisy et al 2016) reported no significant associations (Supplementary Table 3).

Intron 4 variant of *eNOS* (rs869109213) based on a 27 base pair tandem repeat consists of two alleles: allele 4a (with four repeats) and allele 4b (with five repeats). This variant is believed to affect the *eNOS* gene transcription and processing rate. Individuals with carriers of the 4a allele were found to exhibit lower NO levels than 4b/4b homozygous (Zanchi et al. 2000; Mamoulakis et al. 2009; Zintzaras et al. 2009). In this study, rs869109213 was found to be associated with only DN development in both cohorts and no risk could be attributed towards T2D. A similar observation was made by other studies where *eNOS* rs869109213 was found to be significantly associated with DN (Neugebauer et al. 2000; Buraczynska et al. 2004; Bellini et al. 2007; Ahluwalia et al. 2008; Ezzidi et al. 2008; Mehrab-Mohseni et al. 2011). No significant association of rs869109213 with DN was documented in the Brazilian (Santos et al. 2011), Egyptian (Elsisy et al. 2016), Iranian (Rahimi et al. 2013), Chinese (Dong et al. 2007) and German (Degen et al. 2001) populations (Supplementary Table 3).

The *MCP-1* rs1024611 promoter variant is believed to regulate the MCP-1 protein levels. The cells with the homozygous AA genotype produce relatively less amount of MCP-1 compared to the cells with AG or GG genotypes (Rovin and Saxena 1999). rs1024611 G allele compared to the A allele is associated with an increased production of both *MCP-1* transcript and protein (Rovin and Saxena 1999; Fenoglio et al. 2004). *MCP-1* rs1024611 G allele provided a risk towards DN progression in both cohorts. In patients of T2D without DN, rs1024611 G allele conferred a protection for T2D development in cohort-I, whereas, in cohort-II, no association was found. Our results aligned with a previous study in the Asian Indian population, where rs1024611 G allele conferred a risk towards DN progression (Ahluwalia et al. 2009). However, studies on Korean (Moon et al. 2007) and Turkish populations (Karadeniz et al. 2010; Bagci et al. 2015) reported that rs1024611 A allele rather than G allele conferred a risk for nephropathy. In cohort-I, rs1024611 G allele conferred a risk for DN but protection towards T2D development. Reports from Chinese (Jing et al. 2011) and German (Simeoni et al. 2004) populations also revealed that rs1024611 G allele conferred a protection against T2D development. However, some studies also reported no association of rs1024611 with T2D in Japanese (Kouyama

et al. 2008) and with DN in the Korean (Joo et al. 2007) populations (Supplementary Table 3).

Intronic variants are capable of affecting mRNA alternative splicing and may also provide a platform to act as enhancers to increase gene expression (Chorev and Carmel 2012). *MCP-1* rs3917887 (14 bp insertion/deletion) present in intron 1 is considered to disturb the *MCP-1* gene transcriptional activity (Chinoy et al. 2007). *MCP-1* rs3917887 conferred a risk towards both DN and T2D in both cohorts. A study by Ahluwalia et al. (2009) demonstrated the association between *MCP-1* rs3917887 I allele and DN risk. However, observations in the present study were different as in our analysis rs3917887 D allele instead of I allele gave a risk for both T2D and DN development. These differing results may be due to the "flip-flop" phenomenon, which indicates the heterogeneous effect of the same variant due to changes in genetic background or environment (Lin et al. 2007).

Haplotypes provide important information about human evolution and the identification of genetic polymorphisms causing various human traits through LD (Liu et al. 2008). Haplotype combination with risk alleles increased the disease susceptibility in two cohorts. The *eNOS* haplotypes C-a-T and C-b-T provided risk for DN development in both cohorts, however, the risk for T2D was observed only in cohort-II. In *MCP-1*, the G-D haplotype provided a risk for both T2D and DN progression in two populations. While in cohort-I, haplotype G-I gave a protection towards T2D.

Socioeconomic factors have been associated with higher prevalence of diabetes and diabetes-related complications (Connolly et al. 2000; Rabi et al. 2006; Suwannaphant et al. 2017). In this study, both cohorts had uneducated subjects, had lower economic status, smoked and consumed non-vegetarian diet and alcohol. All these factors made the majority of participants susceptible to diabetes and its complications. Previous studies have also demonstrated the association of low education (Suwannaphant et al. 2017) and low income (Rabi et al. 2006; Bird et al. 2015) with diabetes. Therefore, it may be possible that the disease progression in these cohorts may relate to physical inactivity, lifestyle and environmental risk factors for T2D (Connolly et al. 2000; Bird et al. 2015).

DN is a complex disorder attributed to the interaction between multiple genes and environmental factors (Galkina and Ley 2006; Tesch 2010; Dhananjayan et al. 2016). Along with the genetic factors, environmental factors also play a crucial role in disease pathogenesis (Dellamea et al. 2014). It is assumed that the mutual effect of genotype–phenotype interactions is a major component of the predisposition to the disease. In this study, a significant association of *eNOS* and *MCP-1* genotypes with BMI, urea, creatinine and random blood sugar levels was observed. Experimental evidences have suggested that levels of urea, creatinine and glucose levels are involved in increased insulin resistance and promote the progression of renal disease in T2D patients (Neumiller and Hirsch 2015; Kashima et al. 2017; Li et al. 2018; Osman et al. 2018; Xie et al. 2018; Zaman et al. 2018; Davies et al. 2018). Although there are few studies available on the association of *eNOS* and *MCP-1* genotypes with T2D and DN phenotypes, these studies have not deciphered the association of these genotypes with BMI, urea, creatinine and random blood sugar levels (Joo et al. 2007; Hassan et al. 2010; Moguib et al. 2017; Sadati et al. 2018).

   Meta-analysis is an important method for summarizing research findings, for increasing statistical power and for enabling the identification of reliable associations between genetic variants and disease phenotype (Martorell-Marugan et al. 2017). In this study, the meta-analysis revealed that all three *eNOS* variants were associated with DN risk. However, *eNOS* rs869109213 was associated with T2D progression. These differences observed for the two disease groups could be due to the fact that same gene variant can have varied effect on different disease phenotypes (Raina et al. 2015b). This study demonstrated a few noted limitations such as in the era of next-generation sequencing, we used RFLP and ARMS-based PCRs for genotyping due to limited funding. However, RFLP and ARMS PCR assays are found to be quick, cost effective, easy, reliable and with reproducible results (Little 2001; Zhang et al. 2005; Chen et al. 2007; Ota et al. 2009; Tabit 2016). Moreover, the present study did not validate the functional role of the studied genes and also the role of long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) involved in the regulation of expression of these genes. The deeper functional understanding of these genes will help to identify the risk assessment model for these variants and proteins that are associated with T2D and DN aetiology.

Conclusion

Our study is the first systematic study that assessed two ethnically and geographically distinct cohorts from North India with three different groups for a better understanding of *eNOS* and *MCP-1* variants in T2D and nephropathy susceptibility. Our results revealed that all the studied allelic variants are associated with DN risk in both cohorts. Individuals with wild-type allele of these variants may have better probabilities of surviving diabetes-related secondary complications and these alleles may have the nephroprotective effect. However, no association was observed for rs1799983 (in cohort-I), rs2070744 (in cohort-II), rs869109213 (in both cohorts) and rs1024611 (in cohort-II) with T2D cases without DN. These results indicate that probably these gene variants may not directly affect the T2D susceptibility, but in the presence of diabetic milieu these variants increase the risk of progression to DN. These variants may also serve as a valuable genetic markers to identify the diabetic patients who have a high risk of developing nephropathy. The diversity between the two cohorts was confirmed by significant differences in the distribution of demographic and epidemiological parameters, and variation in the effect of the genotypes on the disease susceptibility. However, prospective studies with larger sample size are required for validating the functional role of these identified variants.

Compliance with Ethical Standards

Conflict of interest The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Ethical Approval All the procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Authors and Affiliations

Priyanka Raina[1] ⬤ **· Ruhi Sikka[1] · Himanshu Gupta[2] · Kawaljit Matharoo[1] · Surinder Kumar Bali[3] · Virinder Singh[4] · AJS Bhanwer[1]**

✉ AJS Bhanwer
    ajsbhanwer@gmail.com

[1]   Department of Human Genetics, Guru Nanak Dev University, Amritsar, Punjab 143005, India

[2]   Department of Infection Biology, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, UK

[3]   Department of General Medicine, Government Medical College, Jammu, India

[4]   Dr Virinder Singh Kidney Clinic and Dialysis Centre, Amritsar, Punjab, India